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Chondrodysplasia of gene knockout mice for aggrecan and link protein

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The proteoglycan aggregate of the cartilage is composed of aggrecan, link protein, and hyaluronan and forms a unique gel-like moiety that provides resistance to compression in joints and a foundational cartilage structure critical for growth plate formation. Aggrecan, a large chondroitin sulfate proteoglycan, is one of the major structural macromolecules in cartilage and binds both hyaluronan and link protein through its N-terminal domain G1. Link protein, a small glycoprotein, is homologous to the G1 domain of aggrecan. Mouse cartilage matrix deficiency (*cmd*) is caused by a functional null mutation of the aggrecan gene and is characterized by perinatal lethal dwarfism and craniofacial abnormalities. Link protein knockout mice show chondrodysplasia similar to but milder than *cmd* mice, suggesting a supporting role of link protein for the aggregate structure. Analysis of these mice revealed that the proteoglycan aggregate plays an important role in cartilage development and maintenance of cartilage tissue and may provide a clue to the identification of human genetic disorders caused by mutations in these genes.
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Structure of aggrecan and link protein

The extracellular matrix of cartilage contains two major structures: the fiber structure and the proteoglycan aggregate (Fig. 1) [1]. Whereas collagen fibers give cartilage its tensile strength, proteoglycan aggregates composed of aggrecan [2], hyaluronan (HA), and link protein [3] contribute to water retention and give cartilage its unique gel-like property and resistance to compression. Aggrecan is a large chondroitin sulfate proteoglycan with a total molecular mass of ∼2200 kDa. The core protein is ∼230 kDa in size and contains three globular domains: the N-terminal G1 and G2 domains, the C-terminal G3 domain, plus two glycosaminoglycan attachment domains, keratan sulfate (KS) and chondroitin sulfate (CS). Between G1 and G2, there is another rod-shaped domain termed the interglobular domain. The N-terminal G1 domain binds to both HA and link protein, and their interactions are critical for aggregate formation. The G1 domain can be divided into three loop-like subdomains of A, B, and B′ [4], where the B or B′ subdomain is called the proteoglycan tandem repeat or the link module [5]. Whereas the A subdomain, homologous to the immunoglobulin fold, interacts with link protein [6], a segment of B-B′ binds HA [7]. The C-terminal G3 domain contains a C-type lectin-like domain that binds to various molecules such as tenascin-C [8], sulfated glycolipids [9], and fibulin-1 [10] and -2 [11] . The CS domain contains more than a hundred attachment sites for chondroitin sulfate chains, which enables water retention in the cartilage. In humans, aggrecan CS chains undergo age-related structural changes [12]. Compared with fetal and early postnatal ages, the average CS chain size at skeletal maturity is decreased from ∼20 kDa to ∼8 kDa, and the ratio of 6- to 4-sulfation is increased from ∼0.77 to ∼23. These age-related changes of CS chains may affect not only its hydrated size but also the interactions with other cartilage molecules important for cartilage function. Link protein is a nearly complete copy of the G1 domain of aggrecan and binds to hyaluronan and the G1 domain of aggrecan.

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Cartilage matrix deficiency (*cmd*) mice

Cartilage matrix deficiency (*cmd*) in mice [13] is a natural knockout of the aggrecan gene and is the first example of a mutation of a proteoglycan gene identified in mammals [14]. The homozygotes (*cmd/cmd*) are characterized by dwarfism, a short

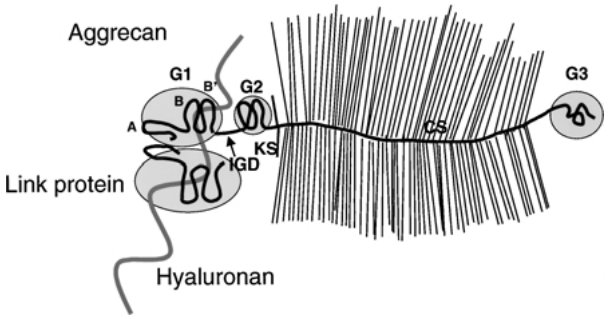

Figure 1. The proteoglycan aggregate composed of aggrecan, hyaluronan, and link protein. Aggrecan contains three globular domains and two glycosaminoglycan attachment domains. G1 consists of three looped subdomains: A, B, and B′. The A loop interacts with link protein, a homologue of G1, whereas a segment of B-B′ interacts with hyaluronan. Interactions of these three molecules are essential for stability of the proteoglycan aggregate.

snout, and a cleft palate (Fig. 2A, B). Whereas heterozygous mice (*cmd*/+) are born normal, homozygous mice (*cmd/cmd*) die shortly after birth due to respiratory failure. The cartilage of homozygous mice appears as tightly packed chondrocytes with little matrix, unlike the extensive matrix seen in normal mice (Fig. 3). The *cmd* aggrecan gene has a single 7-bp deletion in exon 5, which encodes the B loop of the G1 domain [14]. This deletion causes a frame shift resulting in the appearance of a termination codon in exon 6. The potentially truncated 32-kDa polypeptide created by this mutation contains an incomplete proteoglycan tandem repeat structure incapable of HA-binding, but it is not detected. Biochemical and immunological studies demonstrate an absence of aggrecan in the cartilage matrix of *cmd* mice, although the levels of link protein and type II collagen are normal [15]. Analysis of *cmd* growth plate reveals significant differences in the expression of genes encoding cartilage matrix molecules such as link protein, syndecan 3, type II, IX, X, and XI collagens, suggesting that these altered expression patterns may be additional factors contributing to the disrupted cellular architecture of *cmd* growth plate [16].

Heterozygous *cmd* mice

Since heterozygous *cmd* mice have only one normal allele of the aggrecan gene, some metabolic differences in the metabolism of aggrecan may occur and create abnormal phenotypic changes after birth. Although normal at birth, *cmd* heterozygotes exhibit two abnormal phenotypes: a slight dwarfism and late onset spinal misalignment [17]. Approximately 28 days after birth, proportional dwarfism becomes noticeable in the heterozygotes. The most notable abnormality of the *cmd* heterozygotes is a misalignment of the cervical and thoracic spine, which develops approximately one year after birth. They develop a marked lordosis of the cervical spine and kyphosis of the thoraco-lumbar spine. Mice with spinal distortions suddenly acquire a spastic gait disturbance and show decreased movement. They are unable to eat and starve to death within a month following acquisition of the gait disturbance. Thus, the heterozygotes die after 12–15 months, while the wild-type mice live for 2–2.5 years. Histology reveals that the heterozygotes develop degenerative changes of the cervico-thoracic spine. Vertebral bodies are deformed with the disappearance of the apophysis, and the herniated intervertebral disc likely causes spastic gait. In contrast, such specific changes are hardly found in the spines of one-year old wild-type mice. Alcian blue staining in the heterozygotes shows a reduction of glycosaminoglycans. Alcian blue stains tissues surrounding the chondrocytes in the disc cartilage of the heterozygotes, while diffuse staining is observed in the wild type. General features of spinal degeneration are accompanied by pathological changes in both the intervertebral discs and the facet joints. In the *cmd* heterozygotes, pathological changes are found entirely in the intervertebral discs, while the facet joints are apparently normal. These histological findings indicate that the primary lesion lies in the disc and that degeneration characteristic of reactive bone growth does not occur. Because the cervical spine is particularly susceptible to gravitational loading as mice support their head, distortion and herniation may appear at the cervical spine. The knee joint and other cartilaginous tissues are apparently normal in the heterozygotes.

Quantitative reverse transcriptase (RT)-PCR studies reveal reduced levels of aggrecan mRNA in *cmd* heterozygotes and in homozygotes to 81% and 41%, respectively, compared to that in the wild-type mice. Levels of type II collagen mRNA of both the heterozygote and the homozygote are similar to that in the wild-type mice. The levels of chondroitin sulfate in cartilage from 90 day-old *cmd* heterozygous mice are reduced to 87% of the wild-type. Because of the relatively rapid turnover of aggrecan, decreased synthesis of aggrecan may cause significant reduction of its deposition in the tissue. Abnormal phenotypes of the heterozygotes are likely due to the reduced levels of aggrecan expression and deposition. Aggrecan has been shown to play a key role in maintaining the collagen network [18]. Electron microscopy of *cmd* homozygote cartilage shows abnormal collagen fibrils, which display increased diameter, periodic banding patterns, and bundling formations [19]. These results also suggest a role of aggrecan in collagen fibrillogenesis. Similar changes, such as rough fiber distribution with concentric patterns, are found in the disc of the *cmd* heterozygotes. Therefore, reduction of aggrecan may cause fragility both by its own reduced deposition and by affecting collagen fibers.

Spinal misalignment and movement problems involving spastic paralysis in *cmd* heterozygotes resemble spinal paralysis in humans. It is possible that an analogous aggrecan gene defect causes spinal disc herniation or spondylomyelopathy, which are well known as diseases typical for older humans. These findings in *cmd* heterozygotes support aggrecan as a candidate gene predisposing individuals to spinal problems.

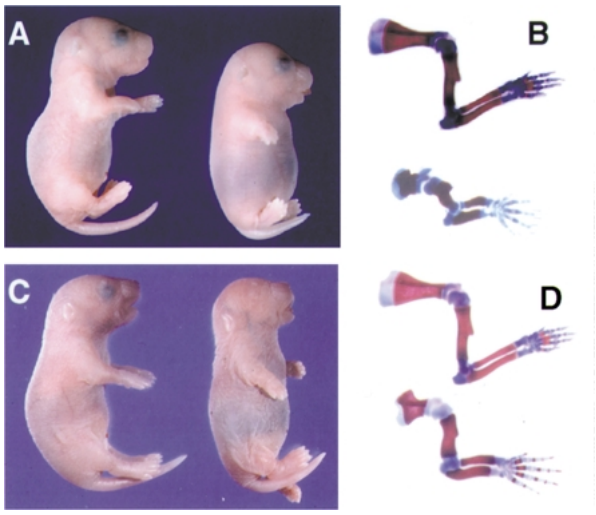

Figure 2. Newborn wild-type, *cmd/cmd*, and *LP*-null mice. Gross phenotype of *cmd/cmd* (A) and *LP*-null (C) are shown compared with the wild-type. Forelimbs stained with alcian blue/alizarin red of *cmd/cmd* (B) and *LP*-null (D) are shown with the wild-type.

Link protein knockout mice

The link protein knockout mice ($LP^{-/-}$) [20] show dwarfism and a flat face, whereas heterozygous mice have no apparent phenotype and are fertile (Fig. 2C, D). Similar to *cmd* mice, most homozygotes (about 93%; 120 out of 129) die shortly after birth due to respiratory failure. Both bones and cartilage are present in newborn ($LP^{-/-}$), although their long bones are shortened. The skulls are small with a shortened antero-posterior axis, although their frontal and parietal bones and occipital squama are normally formed and mineralized. Accordingly, the cranial bones formed through cartilage templates are affected in ($LP^{-/-}$), whereas other cranial bones derived from membranous ossification are normal.

During development, the growth plate normally forms layered zones of chondrocytes representing various differentiation stages, i.e., resting, proliferative, prehypertrophic, and hypertrophic zones. The chondrocytes of the growth plate divide longitudinally to form columns, differentiate into hypertrophic chondrocytes, and undergo apoptosis where the growth plate is invaded by vessels and bone. The skeletons of newborn ($LP^{-/-}$) demonstrate reduced cartilage (Fig. 3). The proliferative and hypertrophic zones are indistinct in newborn ($LP^{-/-}$) and the columns of chondrocytes in the growth plate are disorganized with little bone replacement. Prehypertrophic and hypertrophic cells are intermingled without distinct zones, and the number of hypertrophic chondrocytes is decreased. Among these changes, reduced numbers and sizes of hypertrophic chondrocytes first become apparent at about embryonic day 14.5, suggesting that the lack of link protein primarily affects differentiation from prehypertrophic to hypertrophic chondrocytes. The defects observed in the absence of link protein correlates well with its expression pattern with the highest level in the prehypertrophic zone. Like limb cartilage, chondrocytes of the vertebral bodies are disorganized. By bromo-deoxy uridine incorporation analyses, the absence of link protein apparently does not affect cell proliferation. As these observations are also found in mutant mice of cartilage collagens with chondrodysplasias, they are probably due to distinct compensatory membranous ossification and/or altered signaling of regulatory factors such as parathyroid hormone-related proteins toward osteoplastic status. The level of aggrecan is significantly reduced in the ($LP^{-/-}$) cartilage, which confirms an important role of link protein in the deposition of proteoglycan aggregates. In contrast, the amount

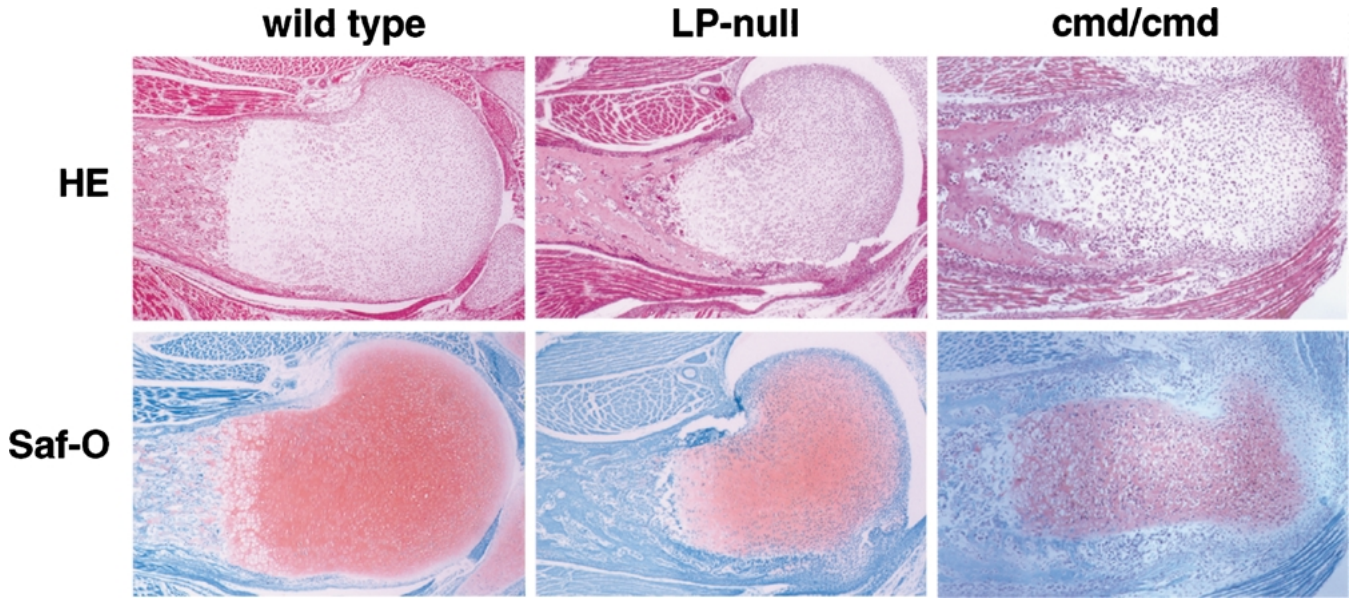

Figure 3. Histology of the wild-type, *LP*-null, and *cmd/cmd* cartilage stained with hematoxylin/eosin or safranin-O/light green.

Figure 4. Spectrum of proteoglycan aggregates obtained by *cmd* and *LP*-null mutations. Phenotypes corresponding to genotypes are shown.

of type II collagen per dry weight of cartilage is similar in ($LP^{-/-}$) and wild-type mice, although the ($LP^{-/-}$) cartilage is smaller in size than the wild-type cartilage. Disorganization of chondrocyte columns is likely due to a reduction of proteoglycan aggregates supporting cartilage structure.

The proteoglycan aggregate apparently plays an important role for chondrocyte differentiation. In ($LP^{-/-}$) cartilage, the expression of Indian hedgehog (IHh) [21], a marker for prehypertrophic chondrocytes that regulates expression of parathyroid hormone-related protein-mediated signal transduction and promotes chondrocyte proliferation, is reduced in the prehypertrophic zone. The parathyroid hormone/parathyroid hormone-related protein receptor [22] is a target of the parathyroid hormone-related protein signaling that regulates differentiation to hypertrophy and is normally expressed in prehypertrophic and hypertrophic chondrocytes and to a lesser extent, in proliferative chondrocytes. In ($LP^{-/-}$) cartilage, its expression is significantly reduced. As the expression of parathyroid hormone-related protein receptor is regulated by parathyroid hormone-related protein, the decreased expression of parathyroid hormone/parathyroid hormone-related protein receptor in ($LP^{-/-}$) cartilage is likely due to the disruption of parathyroid hormone-related protein signaling by adjacent perichondrial cells induced by IHh. Expression of type X collagen, a marker for hypertrophic chondrocytes, is also decreased in ($LP^{-/-}$) growth plate. Decreased deposition of the proteoglycan aggregate by the absence of link protein may alter the levels and distribution of signaling molecules and the localization of chondrocytes that receive them, as well as perturb the signaling pathway for further differentiation of hypertrophic chondrocytes.

Unlike other gene knockout mice with chondrodysplasias [14,23], some of the homozygotes survive the perinatal period. Their shortened trunks, flat faces, and lordosis become obvious with time. Histologically, the growth plate gradually becomes organized. On day 8 after birth, hypertrophic chondrocytes are aligned longitudinally in short columns, and bone invasion of the growth plate is observed. According to column formation, bone cortex and trabeculae become normal.

Although no skeletal disorders have been mapped to the vicinity of the link protein gene locus (assigned as *CRTL1* for humans [24], a certain class of chondrodysplasia, likely inherited in a recessive manner, may be due to a defect of the link protein gene.

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